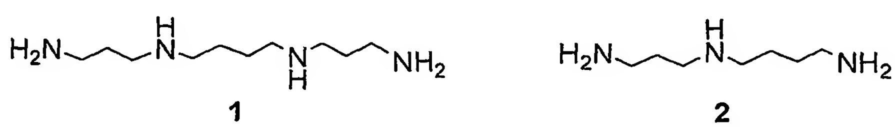
POLYAMINE CONJUGATES WITH ACIDIC RETINOIDS AND PREPARATION THEREOF

FIELD OF THE INVENTION

This invention relates to the preparation of a series of novel polyamine conjugates with vitamin A derivatives which inhibit the ribozyme RNase P and the production of IL-2 and IFN-γ by peripheral blood mononuclear cells in vitro and have potential therapeutic applications in neoplastic, keratinization and inflammatory disorders. In particular, the invention relates to conjugates, obtained from the condensation of linear, conformationally restricted, cyclic and branched polyamines with acidic retinoids, such as *all-trans*-retinoic acid.

BACKGROUND OF THE INVENTION

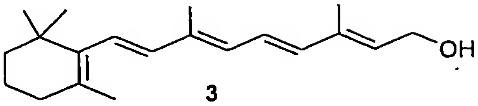
Linear polyamines, like spermine (SPM, **1**) and spermidine (SPD, **2**), and their compounds with other natural products, collectively coined as polyamine conjugates, are widely distributed in living organisms and exhibit interesting biological properties.



In order to determine structure-biological activity relationships and possibly identify lead compounds for the development of polyamine-based pharmaceuticals, a variety of linear, branched, conformationally restricted and cyclic polyamine analogues and conjugates have been synthesized (Blagbrough et al., PHARM. SCI., 3, 223 (1997); Schulz et al., ANGEW. CHEM. INT. END. ENGL., 36, 314 (1997); Papaioannou et al., EUR. J. ORG. CHEM., 1841 (2000) and Kong Thoo Lin et al., SYNTHESIS, 1189 (2000)). Due to their polycationic nature, polyamines interact strongly with nucleic acids and play an important role in their biosynthesis and metabolism. They stabilize DNA conformation and can induce conformation

changes though the formation of intra- or intermolecular bridges. Polyamines cause specific modifications of specialized RNA molecules, stabilize ribonucleases and stimulate the action of ribonucleases and ribozymes. They exert pleiotropic effects on protein synthesis, are essential for normal growth and involved in the differentiation processes of mammalian cells. The concentrations of polyamines and the enzymes responsible for their biosynthesis are notably higher in rapidly proliferating mammalian cells; generally, these concentrations increase in all cells upon induction of differentiation. Polyamines are directly responsible for the increased rate of the macromolecular synthesis occurring during tumour development and growth. Inhibition of the biosynthetic enzymes producing polyamines and of the polyamine uptake system responsible for feeding the cell with exogenous polyamines have emerged as very attractive targets for cancer chemotherapy. Recently, selectively *N*-alkylated polyamines which partially mimic natural polyamine behaviour, inhibit cell growth and are metabolically stable have been developed as novel anticancer agents (for leading references see the review by Papaioannou et al., EUR. J. ORG. CHEM., 1841 (2000)).

The retinoids constitute a large family of organic compounds structurally related to the naturally occurring Vitamin A (retinol, **3**) and analogues, such as retinal (**4**) and *all-trans*-retinoic acid (**5**) and a variety of other synthetic analogues, such as acitretin (**6**), 13-*cis*-retinoic acid (**7**) and 9-*cis*-retinoic acid (**8**). The polyene chain-shortened *all-trans*-retinoic acid analogues **9** and **10** may be also considered as members of this family.



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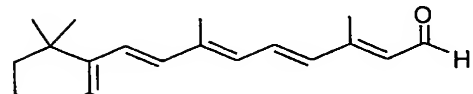

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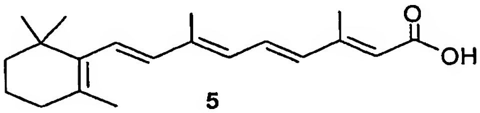

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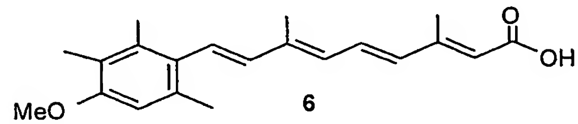

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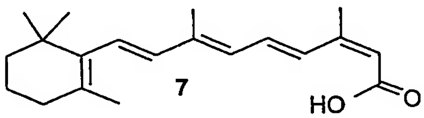

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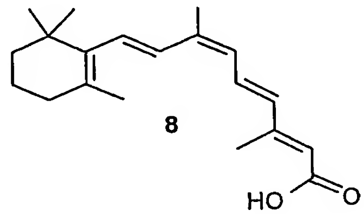

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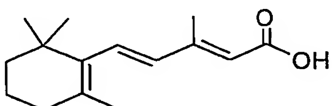

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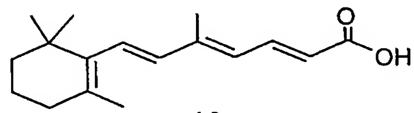

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Retinoids can cause specific biological responses upon binding to and activating special receptors or groups of receptors. Natural and synthetic retinoids play an important role in vision, cell growth, reproduction, proliferation and differentiation of various epithelial or non-epithelial tissues. Although they are already widely used in systemic and topical treatment of various disorders, retinoids reveal a considerable number of side-effects even when used in therapeutic doses. Thus, numerous new retinoid analogues have been synthesized in an attempt to improve the therapeutic index, biological profile and selectivity of these compounds for clinical application in dermatology, oncology, rheumatology and immunology (for general monographies see Sporn, Roberts and Goodman (Eds.), The Retinoids, vol. 1 and 2, Academic Press, Orlando, 1984; Sporn and Roberts, CIBA FOUND. SYMP., 113, 1 (1985); Sporn, Roberts and Goodman (Eds.), The Retinoids : Biology, Chemistry and Medicine, 2nd ed., Raven Press, New York, 1994; Dawson and Okimura (Eds.), Chemistry and Biology of Synthetic Retinoids, CRC Press, Boca Raton, 1990; Packer (Ed.), Methods in Enzymology, Academic Press, vol. 189, part A, 1990 and vol. 190, part B, 1991)). The clinical application of synthetic retinoids in the management of recalcitrant and previously incurable neoplastic, inflammatory and keratinization disorders has introduced a real revolution in

dermatology and other medical fields (Tsambaos, DERMATOSEN, 44, 182 (1996), Muindi, CANCER TREAT. RES., 87, 305 (1996)). By regulating gene expression, retinoids are capable of regulating the differentiation and growth of transformed cells or of inhibiting the malignant transformation of a variety of cells reversing their differentiation (DeLuca, FASEB J., 5, 2924 (1991), Lotan and Glifford, BIOMED. PARMACOTHER., 45, 145 (1991)). In the mechanisms of regulation of gene expression by retinoids certain members of the large family of steroid and thyroid gland hormones receptors are involved, that is nuclear proteins to which retinoids specifically bind (DeLuca, FASEB J., 5, 2924 (1991), Leid et al., TRENDS BIOCHEM. SCI., 17, 427 (1992)). Retinoid receptors have been already isolated and studied (Redfern, PATHOBIOL. 60, 254 (1992), Giguere et al., NATURE, 330, 624 (1987), Petkovich et al., NATURE, 330, 444 (1987)). They act as transcription factors following activation by suitable ligands. Currently, the development of new retinoid-based drugs is based on the synthesis of novel ligands for the retinoic acid receptors RARα,β,γ and RXRα,β,γ and the orphan receptors (Lippman and Lotan, J. NUTR. 130(2S Suppl), 479S (2000)).

It has been recently reported that natural retinoids, like retinoic acid and retinol, as well as synthetic analogues of retinoic acid, e.g. isotretinoin (13-*cis*-retinoic acid), acitretin and the arotinoids Ro 13-7410, Ro 15-0778, Ro 15-1570 and Ro 13-6298 but also other compounds, e.g. calcipotriol, anthralin and their combination, known for their antipsoriatic activity, inhibit the enzyme ribonuclease P (RNase P) (Papadimou *et* al., J. BIOL. CHEM. 273, 24375 (1998), Papadimou *et* al., SKIN PHARMACOL. APPL. SKIN PHYSIOL. 13, 345 (2000), Papadimou *et* al., EUR. J. BIOCHEM. 267, 1173 (2000), Drainas *et al.*, SKIN PHARMACOL. APPL. SKIN PHYSIOL.13, 128 (2000), Papadimou *et* al., BIOCHEM. PHARMACOL. 60, 91 (2000)), which has been isolated and characterized from the slime mold *Dictyostelium discoideum* (Stathopoulos et al.; EUR.J. BIOCHEM. 228, 976 (1995)) and from normal human epidermal keratinocytes.(Drainas et al, unpublished results). These finding advocate the hypothesis that retinoids, in addition to regulating DNA transcription, can also regulate the activity of enzymes playing key-roles in macromolecular biosynthesis, by their implication in post-transcriptional processes, in which binding to the retinoic acid receptors is not

involved. RNase P is responsible for the ripening of the 5' terminus of precursor tRNA molecules. RNase P activity have been found in all pro- and eucaryotic organisms studied so far (Frank and Pace ANNU. REV. BIOCHEM. 67, 153 (1998)). RNase P enzymes are complexes of RNA with proteins and their activity is mainly attributed to their RNA subunit. Several findings indicate that the structure of the RNA subunit is of similar size in pro- and eucaryotic organisms and that the structures of RNase P from different eucaryotic organisms are similar. For these reasons, it appears that RNase P from *D. discoideum* and human epidermal keratinocytes are good models for the identification and development of new inhibitors.

Attaching a polyamine on another bioorganic molecule results in the formation of a polyamine conjugate. Depending on the structure of the non-polyamine moiety, these conjugates are designed to exhibit improved biological activity on particular cellular targets or combine the activities of the constituent molecules. The compounds (conjugates) of the present invention were synthesized in an attempt to combine the biological profiles of polyamines and retinoids. They were all obtained by using as key-step the coupling of the polyamine analogues tabulated in Figure 1 with the retinoids depicted in Figure 2. It was anticipated that the polyamine affinity for nucleic acids, e.g. the RNA part of RNase P, would enforce the binding of natural and synthetic retinoids on the same molecule. Recently, N^1,N^3-diretinoyl-1,3-diaminopropane was synthesized, from 1,3-diaminopropane and retinoyl chloride, as potential antitumour agent but showed low cytostatic activity (Manfredini et al., J. MED. CHEM., 40, 3851 (1997)), whereas cosmetic and/or dermatological compositions consisted of retinol or retinol esters and polyamine polymers were prepared and showed that the polyamine polymers provide superior stabilization to retinol in skin care compositions compared to known products with antioxidant or retinol stabilizing properties (Nguyen et al, US 6,344,206 B1).

Indeed, the conjugates described in the present invention reveal stronger inhibitory effects on RNase P isolated from *D. discoideum* and human epidermal keratinocytes than the parent retinoids or a combination of free polyamines and the corresponding retinoids (Table 1). From the same studies it appears that the more free amino functions in the conjugates are available to interact, the stronger is the

RNase P inhibition (e.g. the spermine conjugate with *all-trans*-retinoic acid is a stronger inhibitor than the corresponding spermidine conjugate). On the other hand, conjugates with two retinoid residues are more active than those with one residue (e.g. spermine bearing two *all-trans*-retinoic acid residues is a stronger inhibitor than spermine bearing only one). In addition, these compounds show an inhibitory effect on the production of IL-2 and IFN-γ by peripheral blood mononuclear cells of healthy human subjects in vitro. In sharp contrast, the currently available retinoids show a stimulatory effect on the production of these cytokines. These results indicate that the compounds described herein have significant potential for clinical application in the treatment of neoplastic, inflammatory and keratinization disorders.

DESCRIPTION OF DRAWINGS

FIGURE 1 : Structures of polyamines used to prepare the conjugates described in the present invention

FIGURE 2 : Structures of retinoids used to prepare the conjugates described in the present invention

FIGURE 3 : General method for the preparation of isolable succinimidyl esters of acidic retinoids

FIGURE 4 : Synthetic schemes for the preparation of N^{α}-mono- and N^{α},N^{ω}-bisamides of linear polyamines with acidic retinoids

FIGURE 5 : Synthetic schemes for the preparation of the N^4,N^9-bisamide of spermine and of all three *N*-monoamides of spermidine with acidic retinoids

FIGURE 6 : Synthetic schemes for the preparation of N^{α},N^{ω}-bisamides of conformationally restricted tetra- and hexa-amines with acidic retinoids

FIGURE 7 : Synthetic schemes for the preparation of polyamides symmetric and asymmetric dimers of spermine and spermidine and of cyclic hexa- and octa-amines and of with acidic retinoids

FIGURE 8 : Double reciprocal plot (1/v versus 1/[pre-tRNA]) for RNase P reaction in the presence of N^1,N^{12}-RA_2-Spermine. The reaction was carried out at the indicated concentrations in the presence or absence of inhibitor. All reactions

were carried out at 37 °C in 20 μl buffer D in the presence of 10% DMSO. (◆) without inhibitor, with N^1,N^{12}-RA_2-Spermine at (■) 3 μM, (▲) 4 μM, (●) 5 μM. *Top panel*: Replot of the slopes of the double reciprocal lines versus inhibitor (I) concentrations.

FIGURE 9 : Effect of N^1,N^{12}-RA_2-SPM on the percentage of CD4+/IL-2+ and CD8+/IL-2+ peripheral blood mononuclear cells.

FIGURE 10 : Effect of N^1,N^{12}-RA_2-SPM on the mean fluorescence intensity (MFI) of CD4+/IL-2+ and CD8+/IL-2+ peripheral blood mononuclear cells.

FIGURE 11 : Effect of N^1,N^{12}-RA_2-SPM on the percentage of CD8+/IFN-γ+ peripheral blood mononuclear cells.

FIGURE 12 : Effect of N^1,N^{12}-RA_2-SPM on the mean fluorescence intensity (MFI) of CD8+/IFN-γ+ peripheral blood mononuclear cells.

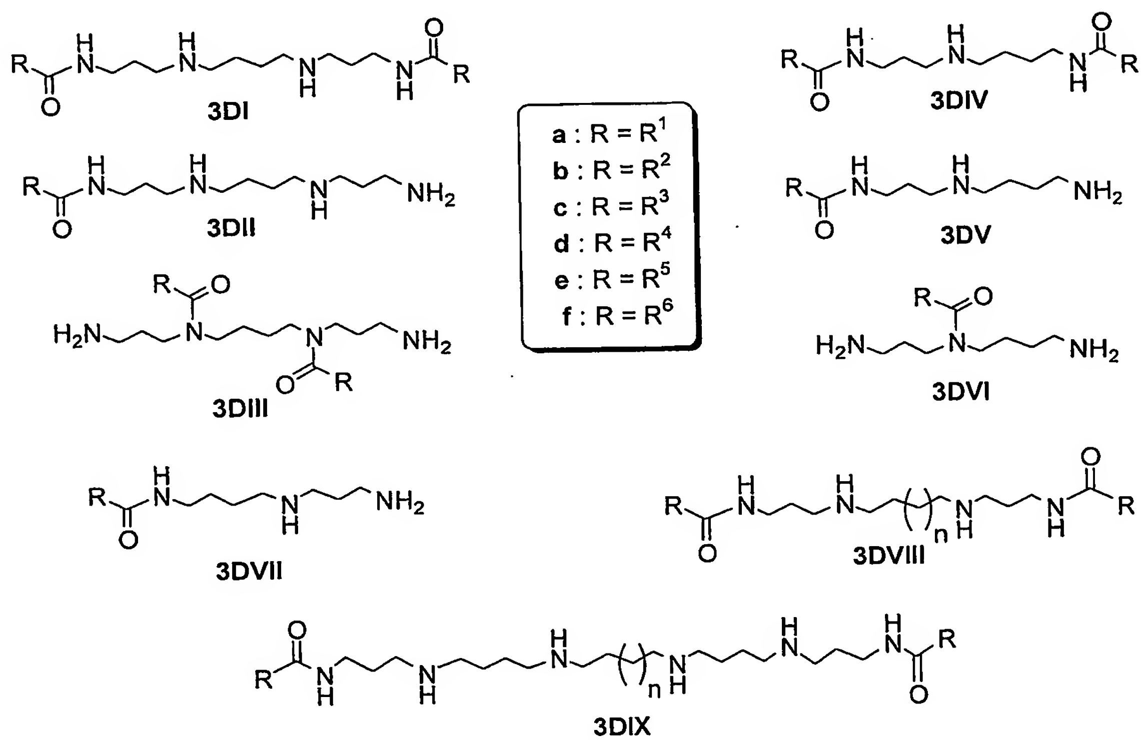
SUMMARY OF THE INVENTION AND DESCRIPTION OF THE PREFERRED EMBODIMENTS

The compounds of the present invention are polyamine conjugates with acidic retinoids, which are prepared through the condensation of either free polyamines or selectively protected polyamines, from those depicted in Figure 1, with either the retinoids directly or the corresponding succinimidyl esters of the acidic retinoids tabulated in Figure 2. The conjugates of the present invention are inhibitors of the enzyme RNase P, isolated from the slime mold *D. discoideum* and human epidermal keratinocytes. The most potent inhibitor among these conjugates has also shown excellent inhibitory effects on the production of IL-2 and INF-γ by peripheral blood mononuclear cells. Thus, it is reasonable to assume that these compounds may be useful in the management of inflammatory disorders.

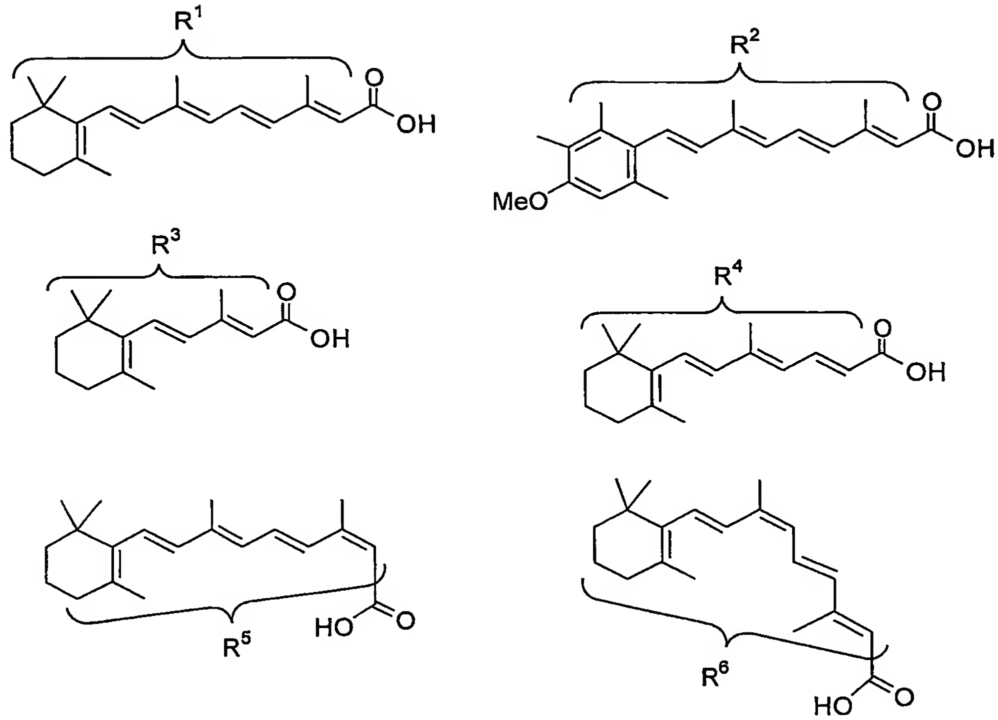
The biological evaluation of the conjugates of the present invention through the examination of their inhibitory activity on RNase P from slime mold *D. discoideum* and human epidermal keratinocytes constitutes a rapid and safe test for evaluation of their potential to modulate the epithelial differentiation and

proliferation and to reverse the malignant transformation of epithelial cells. Thus, alternative laborious tests for retinoid screening based on the retinoic acid receptor (RAR) mediated transcriptional activation (Astrom, BIOCHEM. BIOPHYS. RES. COMMUN., 173, 339 (1990)), or suppression of the expression of an enzyme (Michel, ANAL. BIOCHEM., 192, 232 (1991)), or the induction of a protein RNA (Elder, J. INVEST. DERMATOL. 106, 517 (1996)) become unnecessary.

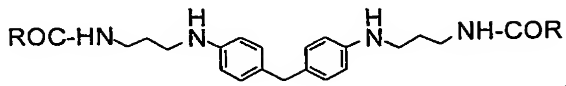
One subfamily of the compounds of the present invention is represented by the following general formulae **3DI-3DIX**, which represent conjugates of linear polyamines, of variable numbers of carbon and nitrogen atoms in the chain, with acidic retinoids.



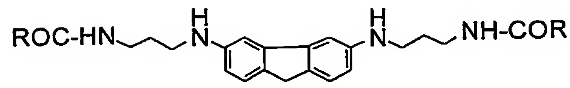
The subscript n in the formula **3DVIII** varies from 2-9 and in the formula **3DIX** from 1-9. The substituent R is one of the following substituents R^1-R^6, preferably R^1.



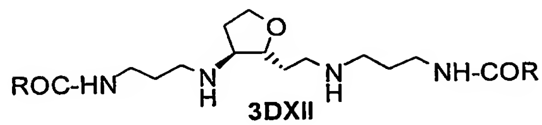
The other subfamily of the compounds of the present invention with the general formulae **3DX-3DXVII**, includes conjugates of conformationally restricted, cyclic and branched (dimeric) polyamines with acidic retinoids. Restriction of conformation in the polyamine moiety is imposed by e.g. aromatic rings incorporated in the chain (conjugates **3DX** and **3DXI**) or heterocyclic rings (conjugates **3DXII**) whereas the cyclic polyamines used are of various ring-sizes and contain different numbers of carbon, nitrogen and oxygen atoms in the ring (conjugates **3DXIII-3DXVI**). In this subfamily, the polyamine moiety is also consisted of symmetric or asymmetric polyamine (spermine and spermidine) dimers (conjugates **3DXVII**). In this category of compounds, the substituent R is one of the above mentioned R^1-R^6, preferably R^1, whereas n is one of the numbers 1, 2 and 7. In compounds **3DXVIIA**, R' is identical to R'' and equal to COR. In compounds **3DXVIIB**, R' is also identical to R'' but equal to $(CH_2)_3$NHCOR. Finally, in compounds **3DXVIIC**, R' is equal to COR and R'' is equal to $(CH_2)_3$NHCOR.



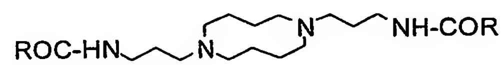
3DX



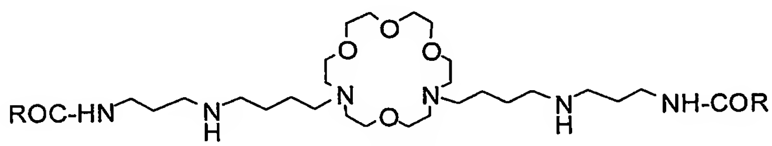
3DXI



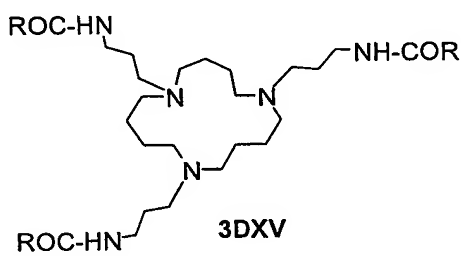
3DXII



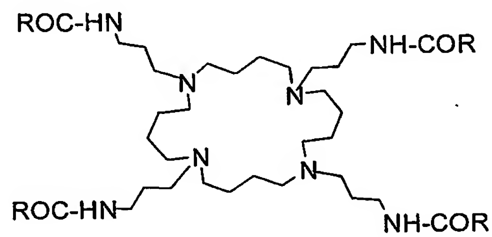
3DXIII



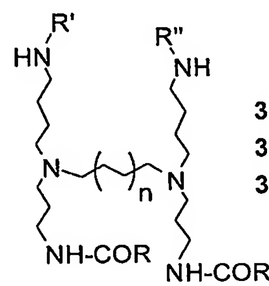
3DXIV



3DXV



3DXVI



3DXVIIA ; R'=R''=RCO
3DXVIIB ; R'=R''=$(CH_2)_3$NHCOR
3DXVIIC ; R'=RCO, R''=$(CH_2)_3$NHCOR

Synthesis

Key-reaction in the synthesis of the polyamine amides described in the present invention is the coupling of an acidic retinoid or activated derivatives of an acidic retinoid with either a free polyamine (direct method) or a suitably protected derivative of a polyamine (indirect method). The acidic retinoids used in this work were either commercially available, e.g. *all-trans*-retinoic acid (ALDRICH), 9- and

13-*cis*-retinoic acid (SIGMA) and acitretin (ROCHE) or synthesized using standard reactions, e.g. the polyene chain-shortened *all-trans*-retinoic acid analogues **9** and **10** depicted in Figure 2. In particular, β-ionylideneacetic acid (**9**) was obtained according to a published protocol (Tietze und Eicher, 'Reaktionen und Synthesen im organisch-chemischen Praktikum', Thieme, New York, 1981, p 445), whereas β-ionylidene-*trans*-crotonic acid (**10**) was synthesized from β-ionylidenethanol (previous reference, p. 446) through a three-steps protocol involving oxidation to the corresponding aldehyde with *o*-iodoxybenzoic acid (IBX) in DMSO (Frigerio et al, J. ORG. CHEM., 60, 7272 (1995)), Wittig reaction with diethyl (ethoxycarbonyl)methylphosphonate and finally saponification. Taking into consideration the sensitivity of retinoids towards strongly acidic reagents, we chose to activate the acidic retinoids in the form of their corresponding 'active' esters with *N*-hydroxysuccinimide (HOSu) which are hydrolytically relatively stable and can be readily purified, if necessary, with flash column chromatography (FCC). In addition, the succinimidyl esters of α,β-unsaturated carboxylic acids react only with the primary amino group of polyamines (Papaioannou et al, TETRAHEDRON LETT., 43, 2593 (2002)). The succinimidyl esters of acidic retinoids (**21**) are simply obtained (Figure 3) by treating the acidic retinoid with HOSu in the presence of the coupling agent *N,N'*-dicyclohexylcarbodiimide (DCC) (see EXAMPLE 1). The succinimidyl esters **21** thus obtained are of sufficient purity to be used in the next step. However, pure samples can be readily obtained through purification with FCC. Esters **21** are then used to acylate the primary amino groups of either the free polyamines (direct method) or polyamines protected at their secondary amino functions with protecting groups, such as 9-fluorenylmethoxycarbonyl (Fmoc) or trifluoroacetyl (Tfa), which can be subsequently removed under basic conditions (indirect method). Examples of both methodologies in the preparation of linear N^{α}-mono(**3DII**)- and N^{α},N^{ω}-diacetylated tetra-amines (**3DI** and **3DVIII**) and N^{α},N^{ω}-diacetylated triamines (**3DIV**) and hexa-amines (**3DIX**) are presented in Figure 4 and detailed under the EXAMPLES 2 and 3. Useful precursors for the indirect methodology are polyamines bearing the triphenylmethyl (trityl, Trt) protecting group at their terminal amino functions, like **22**, **26** and **27**, whose preparation has been described by one of the inventors using the amide approach for the assembly

of the polyamine chain (Papaioannou et al, TETRAHEDRON LETT., 36, 5187 (1995); 39, 5117 (1998); 42, 1579 (2001); 43, 2593 and 2597 (2002) and Papaioannou et al, in 'Drug Discovery and Design : Medical Aspects', J. Matsoukas and T. Mavromoustakos (Eds.), IOS Press, Amsterdam, 2002, in press). These precursors are then routinely protected at their secondary amino function(s) with e.g. the Fmoc group and finally detritylated by a solution of trifluoroacetic acid (TFA) in dichloromethane (DCM). Mono- and/or bisacylation is then performed using one or two equivalents of esters **21**, respectively. Finally, secondary amino group deprotection is carried out using a 20% solution of piperidine (Pip) in DCM, following routine purification of the fully protected intermediates by FCC, if necessary.

The preparation of polyamines acylated at their secondary amino functions by activated acidic retinoid derivatives is exemplified in Figure 5 with the preparation of the spermine conjugates **3DIII** and described in detail under the EXAMPLE 4. Thus, selective trifluoroacetylation of the primary amino functions (O'Sullivan and Dalrympe, TETRAHEDRON LETT., 36, 3451 (1995); Blacklock et al, TETRAHEDRON LETT., 36, 7357 (1995); Krakowiak and Bradshaw, SYNTH. COMMUN., 28, 3451 (1998); Blagbrough et al, TETRAHEDRON, 56, 3439 (2000)) with CF_3CO_2Et followed by acylation of the remaining amino functions with the acidic retinoid in the presence of the powerful coupling agent bromotripyrrolidinophosphonium hexafluorophosphate (PyBrOP) and $^iPr_2NEt$ leads to the fully protected spermine derivatives **28**, from which the projected conjugates **3DIII** are obtained through alkaline hydrolysis. Using the same methodology described in detail under and EXAMPLE 5, the N^4-monoacylated spermidine conjugates **3DVI** were obtained (Figure 5), whereas the other two possible regioisomers **3DV** and **3DVII** became available through the corresponding N^1(**29**)- and N^8(**30**)-tritylspermidines, according to the methodology also described in Figure 5 and detailed under the EXAMPLE 6. The preparation of the former precursor has been already described (Papaioannou et al, TETRAHEDRON LETT., 42, 1579 (2001)) whereas the latter was readily obtained through coupling of the chloride Fmoc-$NH(CH_2)_2COCl$ (Papaioannou et al, TETRAHEDRON LETT., 36, 5187

(1995)) with *N*-tritylputrescine in the presence of $^iPr_2NEt$, followed by routine removal of Fmoc group and finally $LiAlH_4$-mediated reduction.

The preparation of the polyamine bisamides **3DX-3DXIV** incorporating two retinoid residues at their primary amino functions is described in Figure 6 and is identical to the direct preparation of the conjugates **3DVIII.2SuOH** (Figure 4). It involves simple treatment of the tetra-amines **13-15** and **18** and the hexa-amine **16** with two molar equivalents of the succinimidyl esters **21**. In case the free bases are not readily available, the corresponding polytrifluoroacetate salts are used instead and $^iPr_2NEt$ for their *in situ* neutralization (see EXAMPLE 2, direct method B). Syntheses of the polyamines **13-16** and **18**, used as starting materials in these preparation, have been already described by one of the inventors (Papaioannou et al, TETRAHEDRON LETT., 43, 2593 (2002) and Papaioannou et al, in 'Drug Discovery and Design : Medical Aspects', J. Matsoukas and T. Mavromoustakos (Eds.), IOS Press, Amsterdam, 2002,. in press). Finally, the preparation of the polyamine tri(**3DXV**)- and tetra(**3DXVI** and **3DXVIIA-C**)-amides incorporating three and four retinoid residues, respectively, at their primary amino functions is described in Figure 7. These syntheses involve the coupling of the corresponding polyamines **19** and **17** and **20** with three and four molar equivalents, respectively, of succinimidyl esters **21**. The synthesis of polyamines **17** (Papaioannou et al, TETRAHEDRON LETT., 43, 2597 (2002)) and **19** and **20** (Papaioannou et al, TETRAHEDRON LETT., 43, 2593 (2002)) has been also recently described.

Biological Evaluation

Inhibitory activity on RNAse P. We have developed a method by which we can estimate rapidly the biological activity of the polyamine-retinoid conjugates. This method is based on the effect of the polyamine-retinoid conjugates on RNase P activity and is described in detail under the EXAMPLE 7. All synthesized conjugates were screened for their effect on RNase P activity by constructing the dose response curves. From the dose response curves the IC_{50} value (the concentration of conjugate at which the product formation is reduced by 50%) is calculated (Papadimou *et* al., J. BIOL. CHEM. 273, 24375 (1998)), which is a first

and quite reliable measure for the potency of retinoid analogues. The accurate estimation of the inhibitory potency on the RNAse P of the strongest polyamine-retinoid conjugates were elucidated by detailed kinetic analysis of their effect on *D. discoideum* or human epidermal keratinocytes RNase P activity. In order to carry out such analysis, the initial velocity in the presence or absence of the conjugate was determined from the initial slopes of time plots. The data plotted in double reciprocal plots (1/v versus 1/[S]) with increasing concentrations of polyamine-retinoid conjugates and the K_i values (the dissociation constant of the inhibitor (I), where I is a polyamine-retinoid conjugate, with the enzyme) were determined from the replots of the slopes of the double reciprocal plots versus the inhibitor's concentration (Papadimou *et* al., J. BIOL. CHEM. 273, 24375 (1998), Papadimou *et* al., SKIN PHARMACOL. APPL. SKIN PHYSIOL. 13, 345 (2000)). These replots lead to the graphical determination of K_i values from the negative intercept of the line with the I-axis. Figure 8 shows the double reciprocal plot and the slope replot for N^1,N^{12}-bisretinoylspermine. Similar plots were constructed for all conjugates. The K_i value is a very good measure for the accurate potency of the polyamine-retinoid conjugates. The K_i values of the effect of the most potent polyamine-retinoid conjugates on *D. discoideum* RNase P activity are presented in Table 1, below, in comparison to known natural and synthetic retinoids and arotinoids. Similar K_i values were obtained with normal human epidermal keratinocytes RNase P.

Anti-inflammatory activity. Preliminary experiments were performed to determine the optimum conditions for IL-2 and IFN-γ detection intracellularly (data not shown), which mainly involved determination of brefeldin, ionomycin and PMA concentrations and duration of the incubation period and of the brefeldin presence. It was determined that the addition at the initiation of the incubation period of 5 ng/ml of PMA in combination with 250 ng/ml of ionomycin, as well as the addition for the last 2 h of a 4 h incubation period of 5 μg/ml of brefeldin, were accompanied by a peak release of both cytokines in the intracellular space.

PBMC incubation in the presence of PMA/ionomycin was accompanied in all experiments by a significant up-regulation of the IL-2 and IFN-γ expression compared to the unstimulated cultures. This was manifested on both CD4+ and

CD8+ lymphocytes and was evidenced both as an up-regulation of the lymphocyte percentage, as well as an upregulation of the fluorescence intensity. Furthermore, it was observed that CD4+ lymphocytes were the major producers of IL-2, whereas CD8+ lymphocytes were mostly producing IFN-γ.

Addition of a polyamine-retinoid conjugate, e.g. N^1,N^{12}-RA_2-SPM, at the initiation of the culture, at concentrations 10^{-4}, 10^{-5} and 10^{-6} M, had a variable effect on PMA/ionomycin-induced IL-2 levels (see EXAMPLE 8). The highest concentration of the conjugate (10^{-4} M) caused a decrease in the percentage of CD4+/IL-2+ and CD8+/IL-2+ cells, as well as in the intensity of fluorescence, whereas the other two concentrations had a minimal or no effect. At a concentration of 10^{-4} M, the polyamine-retinoid conjugate induced a decrease in CD4+/IFN-γ+ cell intensity of fluorescence and percentage of CD8+/IFN-γ+ cells, whereas the other two lower concentrations revealed a minimal or no effect (Figures 9-12)

Table 1. Selected equilibrium constants derived from primary and secondary plots.

Retinoids	K_i^a (μM)	**Polyamine conjugates with retinoids (new compounds)**	K_i^a (μM)
Retinol	1,475	*N*[1]-RA-Spermine (**3DIIa**)	2.4
all-trans-Retinoic acid (RA)	15	N^1,N^{12}-RA_2-Spermine (**3DIa**)	0.5
Isotretinoin	20	N^4,N^9-RA_2-Spermine (**3DIIIa**)	1.1
Acitretin (Aci)	8	N^1-Aci-Spermine (**3DIIb**). 3 HCl	2.45
Ro 13-7410	45	N^1,N^{12}-Aci_2-Spermine (**3DIb**). 2 HCl	0.95
Ro 15-0788	2,800		
Ro 15-1570	3,600		
Ro 13-6298	4,350		
RA + Spermine	15		

[a]The K_i values are calculated from the negative intercept of the slope replots (see Figure 8)

EXAMPLES

Experimental

Capillary melting points were taken on a Büchi SMP-20 apparatus and are uncorrected. IR spectra were recorded as KBr pellets or with neat oily samples on a Perkin-Elmer 16PC FT-IR spectrophotometer. ^{1}H-NMR spectra were obtained at 400.13 MHz MHz, on a Bruker Avance 400 DPX spectrometer. Electron-Spray Ionization (ESI) mass spectra were obtained on a Micromass-Platform LC spectrometer for solutions of the measured compounds in MeOH. Microanalyses were performed on a Carlo Erba EA 1108 Elemental Analyzer. All new compounds gave satisfactory microanalytical data to within ± 0.3 of the calculated values. Flash Column Chromatography (FCC) was performed on Merck silica gel 60 (230-400 mesh) and Thin layer Chromatography (TLC) on Merck silica gel F_{254} films (0.2 mm) precoated on aluminium foil. The solvent or solvent systems used were : (A) PhMe/EtOAc (95:5), (B) PhMe/EtOAc (9:1), (C) PhMe/EtOAc (7:3), (D) PhMe/EtOAc (1:1), (E) CH_2Cl_2/MeOH (9:1), (F) $CHCl_3$/MeOH (9:1), (G) $CHCl_3$/MeOH/conc. NH_3 (7:3:0.3), (I) $CHCl_3$/MeOH/conc. NH_3 (6:4:0.4), (J) $CHCl_3$/MeOH/conc. NH_3 (5:5:0.5). Spots were visualized with UV light at 254 nm and ninhydrin. All solvents used were dried according to standard procedures prior to use. Experiments involving retinoids were routinely conducted under an atmosphere of Ar and with protection from light. Drying of solutions was effected with anhydrous Na_2SO_4, whereas evaporation of the solvents was performed under reduced pressure in a rotary evaporator at a bath temperature not exceeding 40 °C.

The examples below are given so as to illustrate the practice of this invention. They are not intended to limit or define the entire scope of the invention.

EXAMPLE 1

Preparation of succinimidyl retinoate (**21a**)

To an ice-cold solution of *all-trans*-retinoic acid (**5**) (3.00 g, 10 mmol) in dry THF or DMF (30 ml) was added sequentially HOSu (1.72 g, 15 mmol) and DCC (2.48 g, 12 mmol) and the resulting mixture was stirred for an additional 30 min at 0 °C and for overnight at ambient temperature. The precipitated DCU was filtered off and

washed several times with EtOAc. The combined filtrates were washed sequentially with an ice-cold 5% aqueous (aq.) $NaHCO_3$ solution, H_2O and twice with brine. Drying, followed by filtration and evaporation of the solvent left a residue. FCC of the residue using as eluant the solvent system A, pooling the fractions containing pure product, evaporation and trituration with Et_2O gave 3.38 g (85%) of yellowish crystalline ester **21a** following overnight refrigeration. R_f(A) : 0.24. M.p. : 173-76 °C. FT-IR (cm^{-1}) : 1758, 1732, 1626, 1595, 1574, 1558. 400 MHz ^{1}H-NMR ($CDCl_3$) : δ 7.152 (1H, dd, *J* 11.6 and 15.2 Hz, H-6), 6.352 (2H, d, *J* 15.2 Hz, H-5 and H-11), 6.176 (1H, d, *J* 16.0 Hz, H-10), 6.166 (1H, d, *J* 10.3 Hz, H-7), 5.954 (1H, s, H-2), 2.852 (4H, br. S, H-2' and H-3'), 2.382 (3H, s, H-4), 2.030 (3H, s, H-9), 2.030 (2H, m, H-14), 1.722 (3H, s, H-20), 1.613 (2H, m, H-15), 1.472 (2H, m, H-16), 1.035 (6H, s, H-18 and H-19) ppm. Elemental analysis based on $C_{24}H_{31}NO_4$ ((Calc.) Found) : C (72.52) 72.69; H (7.86) 7.72; N (3.52) 3.28.

EXAMPLE 2

Preparation of N^1,N^{12}-bisretinoylspermine (**3DIa**)

Direct method A :

To an ice-cold solution of spermine (**1**) (0.55 g, 2.7 mmol) in dry CH_2Cl_2 (25 ml) was added ester **21a** (1.99 g, 5 mmol). The resulting solution was stirred for an additional hour at 0 °C and then placed in the refrigerator for overnight. The precipitated product was filtered off, washed on the filter with ice-cold CH_2Cl_2 and dried under reduced pressure to give 2.02 g (80 %) of the bishydroxysuccinimidate salt of **3DIa** as a yellow solid.

R_f(I) : 0.40 (free base) and 0.13 (HOSu). M.p. : 149-52 °C. FT-IR (cm^{-1}) : 3430, 3317, 1674, 1647. ESI-MS (m/z) : 768.74 (*M*H), 767.75 (*M*), 485.56 (*R^1CO-SPM*+H), 580.02 (*M*H-$C_{14}H_{20}$), 384.72 (*M*H_2/2). 400 MHz ^{1}H-NMR (d_6-DMSO) : δ 8.040 (2H, uresolved t, N*H*CO), 6.912 (2H, dd, *J* 12.1 and 14.2 Hz, H-6), 6.33-6.15 (8H, m, H-5, H-7, H-10, H-11), 5.840 (2H, s, H-2), 5.780 (4H, s, H_2N^+), 3.150 (4H, unresolved q, H-1'), 2.529 (16H, m, H-3', H-4', H-2'', H-3''), 2.290 (6H, s, H-4), 2.024 (4H, unresolved t, H-14), 1.976 (6H, s, H-9), 1.704 (6H, s, H-20), 1.598 (8H, m, H-15, H-2'), 1.474 (8H, m, H-16, H-5'), 1.032 (12H, s, H-18, H-19) ppm.

Elemental analysis based on $C_{58}H_{88}N_6O_8$ ((Calc.) Found) : C (69.85) 69.56; H (8.89) 8.97; N (8.43) 8.62.

Direct method B :

When $CHCl_3$ was used as the reaction solvent, complete solution of reactants and products was observed. The reaction was found complete (by TLC) within 30 min at 0 °C and then it was worked-up by diluting the resulting solution with EtOAc, washing sequentially twice with a 5% aq. $NaHCO_3$ solution and twice with water. The organic phase was then dried and evaporated to leave crude product, which was purified by FCC using as eluant the solvent system I to give pure free **3DIa**, as a yellow powder, in75% yield.

Alternatively and in order to facilitate the work-up and purification by FCC procedures, after the diacylation of spermine, in situ protection of the remaining free secondary amino functions with the trifluoroacetyl(Tfa) group, by treating the reaction mixture with trifluoroacetic anhydride (Tfa_2O) in the presence of Et_3N for 5 min at 0 °C και for 30 min at ambient temperature, takes place. Then, the fully protected product is subjected initially to purification with FCC and finally to removal of the temporary protecting groups by treating with K_2CO_3 at refluxing $MeOH/H_2O$ (6:0.5) for 30 min, giving the free product **3DIa** in 68% total yield.

Indirect method :

To an ice-cold solution of N^1,N^{12}-Trt_2-SPM (**22a**; n=1) (2 g, 3 mmol) in dry CH_2Cl_2 (30 ml) was added sequentially $^iPr_2NEt$ (1.4 ml, 8 mmol) and Fmoc-OSu (2.2 g, 8 mmol). After 30 min at ambient temperature, the reaction mixture was diluted with CH_2Cl_2 (100 ml) and washed sequentially twice with a 5% aq. $NaHCO_3$ solution and twice with water. Drying and evaporation of the solvent left a residue which was subjected to FCC using as eluant the solvent system B to give 3.26 g of pure product N^4,N^9-$Fmoc_2$-N^1,N^{12}-Trt_2-SPM as a foam. This was then treated with a solution (20 ml) of trifluoroacetic acid (TFA) in CH_2Cl_2 (1:4) for 30 min at 0 °C. Evaporation of the solvent left a residue which was triturated with Et_2O/hexane (1:1) and refrigerated overnight. The supernatant liquid was poured off and the residue was subjected to FCC using as eluant the solvent system E to give 1.8 g (69% yield based on **22a**) of the bistrifluoroacetate salt of N^4,N^9-$Fmoc_2$ –SPM (**23a**) as a foam.

To an ice-cold solution of **23a** (0.88 g, 1 mmol) in 2 ml DMF/$CHCl_3$ (1:1) was added sequentially $^iPr_2NEt$ (0.7 ml, 4 mmol) and 'active' ester **21a** (0.79 g, 2 mmol). After 1 h at ambient temperature, the reaction mixture was diluted with 50 ml EtOAc and washed once with a 5% aq. $NaHCO_3$ solution and twice with water. Drying, evaporation and FCC with the eluant D gave the intermediate **24a**. This was then treated with a solution (10 ml) of piperidine (Pip) in CH_2Cl_2 (1:4) for 15 min at ambient temperature. Evaporation of the solvent, trituration of the residue with Et_2O, refrigeration and finally filtration gave 0.57 g (74%) of **3DIa**.

EXAMPLE 3

Preparation of N^1-retinoylspermine (3DIIa)

To an ice-cold solution of **23a** (0.88 g, 1 mmol) in 2 ml DMF/$CHCl_3$ (1:1) was added $^iPr_2NEt$ (0.4 ml, 2.3 mmol) and then 'active' ester **21a** (0.32 g, 0.8 mmol) in small portions within 1 h. After additional 30 min at ambient temperature, the reaction mixture was diluted with 50 ml EtOAc and washed once with a 5% aq. $NaHCO_3$ solution and twice with water. Drying, evaporation and FCC of the residue with the eluant F gave the intermediate **25a** with R_f (F) 0.15. This was then treated with a solution (10 ml) of Pip in CH_2Cl_2 (1:4) for 30 min at ambient temperature. Evaporation of the solvent under reduced pressure, trituration of the residue with Et_2O and refrigeration gave a precipitate. Finally, filtration and evaporation of the filtrate gave 0.18 g (47%) of **3DIIa** as a yellowish foam. ESI-MS (m/z) : 485.86 (*M*H).

It should be noted that in the above described reaction of compound **23a** with ester **21a**, considerable amounts of the corresponding diacylated spermine were also formed, but this by-product is easily separated from the monoacylated spermine derivative **25a**, during the afore mentioned FCC. The trishydrochloride salt of **3DIIa** was also obtained, as a yellowish powder, by triturating an ice-cold solution of the free base in MeOH with an ice-cold solution of 1.2 N solution of gaseous HCl in anhydrous MeOH, followed by immediate precipitation of the thus formed salt with Et_2O.

EXAMPLE 4

Preparation of N^4,N^9-bis(*all-trans*-retinoyl)spermine (**3DIIIa**)

To an ice-cold solution of the bistrifluoroacetate salt of N^1,N^{12}-bistrifluoroacetylspermine (0.32 g, 0.5 mmol) in anhydrous $CHCl_3$ (1 ml) was added $^iPr_2NEt$ (0.7 ml, 4 mmol) and a mixture consisted of *all-trans*-retinoic acid (0.3 g, 1 mmol) and PyBrOP (0.6 g, 1.28 mmol) in small portions over a period of 1 h. After 30 min at ambient temperature, the reaction mixture was diluted with $CHCl_3$ and washed sequentially with an ice-cold 5% aq. $NaHCO_3$ solution (twice) and water (twice). Drying and evaporation left a residue from which pure intermediate **28a** ($R=R^1$) was obtained through FCC using the solvent system D as eluant. Intermediate **28a** was dissolved in 4 ml MeOH and treated with 0.4 ml of a 4.75 N aq. NaOH solution for 2 h at ambient temperature. MeOH was then removed under reduced pressure and the residue was taken up in 20 ml water and extracted twice with CH_2Cl_2. The combined organic layers were washed twice with brine, dried and evaporated to leave 0.16 g (40%) of pure **3DIIIa** as a yellow powder. R_f(J) : 0.26. FT-IR (cm^{-1}) : 3434, 1620. ESI-MS (m/z) : 790.30 (*M*Na), 768.31 (*M*H). Elemental analysis based on $C_{58}H_{88}N_6O_8$ ((Calc.) Found) : C (69.85) 70.08; H (8.89) 8.60; N (8.43) 8.21.

EXAMPLE 5

Preparation of N^4-acitretinoylspermidine (**3DVIb**)

A solution of spermidine (**2**) (0.73 g, 5 mmol) and ethyl trifluoroacetate (2 ml, 17,5 mmol) in acetonitrile (15 ml) containing 0.11 ml (6 mmol) H_2O was refluxed for overnight and then the solvent evaporated to leave 2.1 g (92% yield) of the monotrifluoroacetate salt of N^1,N^8-bistrifluoroacetylspermidine as a foam, which was used as such into the next step. Thus, to an ice-cold solution of this salt (0.50 g, 1.1 mmol) in anhydrous DMF (1.4 ml) and $CHCl_3$ (1 ml) was added $^iPr_2NEt$ (0.7 ml, 4 mmol) followed by acitretinoin (0.34g, 1.06 mmol) and PyBrOP (0.82 g, 1.76 mmol). The reaction mixture was stirred at 0 °C for 30 min and at ambient temperature for overnight. Then diluted with EtOAc and washed once with an ice-cold 5% aq. $NaHCO_3$ solution and twice with cold H_2O, dried and evaporated to leave a residue. From this residue, 0.60 g (86%) of the fully protected **3DVIb** was

obtained as a yellow oil after FCC purification using as eluant the solvent system D. R_f(D) 0.26. The product had ESI-MS (m/z) : 668.23 (*M*Na), 645.92 (*M*H). Fully protected **3DVIb** (0.60 g, 1 mmol) was dissolved in MeOH (60 ml) and H_2O (6 ml) and K_2CO_3 (0.52 g, 4 mmol) were added and the resulting mixture was refluxed for 90 min. Then filtered hot and concentrated to dryness. The residue was subjected to FCC, using the solvent system G as eluant, and the fractions with R_f(G) 0.12 were pooled and evaporated to leave pure product **3DVIb** (0.38 g, 83%) as a yellowish foam. The product had ESI-MS : 454.33 (*M*H).

EXAMPLE 6

Preparation of N^8-acitretinoylspermidine (**3DVIIb**)

To an ice-cold solution of N^8-Trt-SPD (**30**) (0.5 g, 1.3 mmol) was added $^iPr_2NEt$ (0.5 ml, 2.6 mmol) and Fmoc-OSu (0.5 g, 2.6 mmol). After 30 min at 0 °C, the reaction mixture was diluted with EtOAc and washed once with an ice-cold 5% aq. $NaHCO_3$ solution, then H_2O and finally brine and dried. Filtration, evaporation and FCC of the residue using the solvent system B as the eluant, gave pure product which was immediately treated with 10 ml of a solution of TFA in CH_2Cl_2 (3:7) for 30 min at 0 °C. Solvent evaporation, trituration of the residue with Et_2O and rejection of the supernatant liquid left 0.41 g (45% yield) of the trifluoroacetate salt of N^1,N^4-$Fmoc_2$-SPD. R_f(E) 0.21. ESI-MS (m/z) : 704.64 (*M*H).

To an ice-cold solution of this salt (0.34 g, 0.48 mmol) in DMF (2 ml) was added $^iPr_2NEt$ (0.2 ml, 1.2 mmol) and the 'active' ester **21b** (0.2 g, 0.48 mmol). After 1 h at 0 °C and 1 h at ambient temperature, the reaction mixture was diluted with EtOAc and washed twice with an ice-cold 5% aq. $NaHCO_3$ solution and twice with H_2O, dried and evaporated to dryness. The residue was subjected to FCC, using as eluant the solvent system C to give 0.31 g (72% yield) of the fully protected **3DVIIb** as a yellowish foam. R_f(B) 0.27. ESI-MS (m/z) : 899.08 (*M*H). This intermediate (0.3 g, 0.33 mmol) was subsequently treated with a 20% solution of Pip in CH_2Cl_2 for 2 h at ambient temperature. The solvent was then evaporated and the residue was triturated with Et_2O. The supernatant liquor was discarded and this procedure was repeated. Finally, drying of the residue left 0.14 g (93%) of pure product **3DVIIb** as a yellow foam. ESI-MS (m/z) : 455.02 (*M*H).

EXAMPLE 7

Biological evaluation of compounds as RNase P inhibitors

RNase P assays were carried out at 37°C in 20μl buffer D (50 mM Tris/HCl pH 7.6, 10 mM NH_4Cl, 5 mM $MgCl_2$ and 5 mM dithiothreitol) if RNase P isolated from *D. discoideum* was used (Stathopoulos et al.; EUR. J. BIOCHEM. 228, 976 (1995)) or buffer K (50 mM Tris/HCl pH 7.5, 100 mM NH_4Cl, 5 mM $MgCl_2$ and 5 mM dithiothreitol) if RNase P isolated from human epidermal keratinocytes was used (Drainas et al, unpublished data), containing 2-5 fmol pre-$tRNA^{ser}$ substrate (an *in vitro* labeled transcript of the *Schizosaccharomyces pombe* $tRNA^{ser}$ gene *supSI*) and 1.3 μg protein from the RNase P fraction. Stock solutions of retinoids (natural or synthetic), are prepared in 100% dimethylosulfoxide (DMSO). When retinoids are used, enzyme assays are carried out in the presence of 10% DMSO. The reactions were stopped by addition of 5 μl stop dye (80% formamide, 50 mM EDTA, 0.1 % bromophenol blue, 0.1 % xylene cyanol). Reaction products were resolved on a denaturing 10% polyacrylamide/8M urea gel and visualized by autoradiography without drying. Activity was quantified by Cerenkov counting of excised gel slices.

EXAMPLE 8

Biological evaluation of compounds as anti-inflammatory agents

For the purpose of the study, peripheral blood mononuclear cells (PBMC) from ten healthy volunteers (age 33-52 yrs) were incubated in 1 ml volume (RPMI 1640/10% FCS) for varying time periods at 37° C in a CO_2 (5%) incubator in the presence or absence of PMA (Sigma, St Louis, MO), ionomycin (Sigma, St Louis, MO) and/or the polyamine-retinoid conjugate (10^{-4}, 10^{-5}, 10^{-6} M) as well as a protein transport inhibitor, brefeldin (Sigma, St Lous, MO) to prevent cytokine secretion in the extracellular space.

Following this incubation, PBMC were stained with either an anti-CD4-FITC or anti-CD8-FITC monoclonal antibody (Beckton Dickinson Hellas, Athens, Greece) in a PBS buffer containing 0.5% BSA and 0.01% NaN_3, and were subsequently fixed with paraformaldehyde and incubated overnight. In the next step, fixed PBMCs were washed and resuspended in a PBS buffer containing 0.5% BSA, 0.5%

saponin (Sigma, St Louis, MO) (to permeabilize cells) and 0.01% NaN_3. Fixed PBMC were subsequently stained with an anti-IL-2/PE or anti-IFN-γ/PE monoclonal antibody (Diaclone, Besancon, France) and analysed for intracellular expression of IL-2 or IFN-γ and membrane expression of the CD4 or CD8 antigens in a FACSCAN flow cytometer. Throughout this step, saponin-supplied PBS was used, since its permeabilisation effect is reversible.

The immunofluorescence cut-off was set up in unstimulated cultures as background and results were expressed either as the percentage of CD4/IL-2+ and CD8/IL-2+ (Figure 9) and CD8/IFN-γ^+ and CD4/IFN-γ^+ (Figure 11) cells, or alternatively as their mean fluorescence intensity (Figures 10 and 12, respectively).

It is to be understood that, while the foregoing invention has been described in detail by way of illustration and example, numerous modifications, substitutions and alterations are possible without departing from the spirit and scope of the invention as described in the following claims.